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The Viral Replication Complex Is Associated with the Virulence of Newcastle Disease Virus▽

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Virulent strains of Newcastle disease virus ([NDV] also known as avian paramyxovirus type 1) can be discriminated from low-virulence strains by the presence of multiple basic amino acid residues at the proteolytic cleavage site of the fusion (F) protein. However, some NDV variants isolated from pigeons (pigeon paramyxovirus type 1 [PPMV-1]) have low levels of virulence, despite the fact that their F protein cleavage sites contain a multibasic amino acid sequence and have the same functionality as that of virulent strains. To determine the molecular basis of this discrepancy, we examined the role of the internal proteins in NDV virulence. Using reverse genetics, the genes encoding the nucleoprotein (NP), phosphoprotein (P), matrix protein (M), and large polymerase protein (L) were exchanged between the nonvirulent PPMV-1 strain AV324 and the highly virulent NDV strain Herts. Recombinant viruses were evaluated for their pathogenicities and replication levels in day-old chickens, and viral genome replication and plaque sizes were examined in cell culture monolayers. We also tested the contributions of the individual NP, P, and L proteins to the activity of the viral replication complex in an *in vitro* replication assay. The results showed that the replication proteins of Herts are more active than those of AV324 and that the activity of the viral replication complex is directly related to virulence. Although the M protein affected viral replication *in vitro*, it had only a minor effect on virulence.

Newcastle disease is a severe infectious disease of birds caused by Newcastle disease virus (NDV), or avian paramyxovirus type 1 (APMV-1). NDV is classified in the genus *Avulavirus* of the family *Paramyxoviridae* (32) and has a single-stranded, negative-sense RNA genome consisting of six genes in the order 3′-NP-P-M-F-HN-L-5′ (28) that encode at least seven proteins: the nucleocapsid protein (NP), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the hemagglutinin-neuraminidase protein (HN), and the polymerase protein (L). During P gene transcription, an additional, nonstructural protein (V) is produced by means of mRNA editing (55).

The M, F, and HN proteins are associated with the viral envelope. The F and HN proteins mediate entry and release, and the M protein is involved in the morphogenesis and budding of NDV (28). The V protein is involved in interferon antagonism (42). The NP protein encapsidates the RNA genome to form the nucleocapsid, which serves as the template for viral transcription and replication. The P protein is essential for viral RNA synthesis and has multiple roles (10, 12). It forms separate complexes with the NP and L proteins and the nucleocapsid (22). Transcription of the viral genomic RNA occurs by way of the viral polymerase (P-L complex); the catalytic activities of the polymerase are functions of the L protein, and the P protein is responsible for the binding of the P-L complex to the nucleocapsid. Once sufficient viral proteins are generated, NP starts to bind to the leader chain, a process in which the P protein acts as a chaperone to deliver NP to the nascent RNA (11). The NP-P complex is believed to regulate the switch from transcription to replication (59), but several findings also show an important role for the M protein in this process. Because the M protein associates with the nucleocapsid (19, 29, 56), it may also affect transcription and/or replication (17, 19, 25, 38). The P-L complex is responsible for genome replication, i.e., the synthesis of full-length plus-strand antigenomic RNA, which in turn serves as the template for the synthesis of minus-strand genomic RNA, which is ultimately packaged into progeny virions. The L protein performs post-transcriptional modification activities such as capping, methylation, and polyadenylation of mRNAs (47, 53). The NP, P, and L proteins together constitute the viral replication complex (28).

Based on the mean times in which they kill inoculated chicken embryos and their virulences for day-old chickens, NDV strains can be categorized into one of four pathotypes, i.e., nonvirulent, lentogenic (low virulence), mesogenic (intermediate), or velogenic (highly virulent) (3). Cleavage of the F protein is required for the initiation of infection and is the major virulence determinant. The cleavage site of the F protein of virulent NDV strains contains multiple basic amino acid residues and is recognized by ubiquitous intracellular furin-like proteases, resulting in a systemic infection. The cleavage site of the F protein of low-virulence strains does not contain these multiple basic amino acids and is recognized by extracellular trypsin-like proteases found in a limited number of tissues, predominantly in the respiratory and intestinal tracts (36, 37), thereby limiting the replication of low-virulence strains to these tissues.

Pigeon paramyxovirus type 1 (PPMV-1) strains are variant

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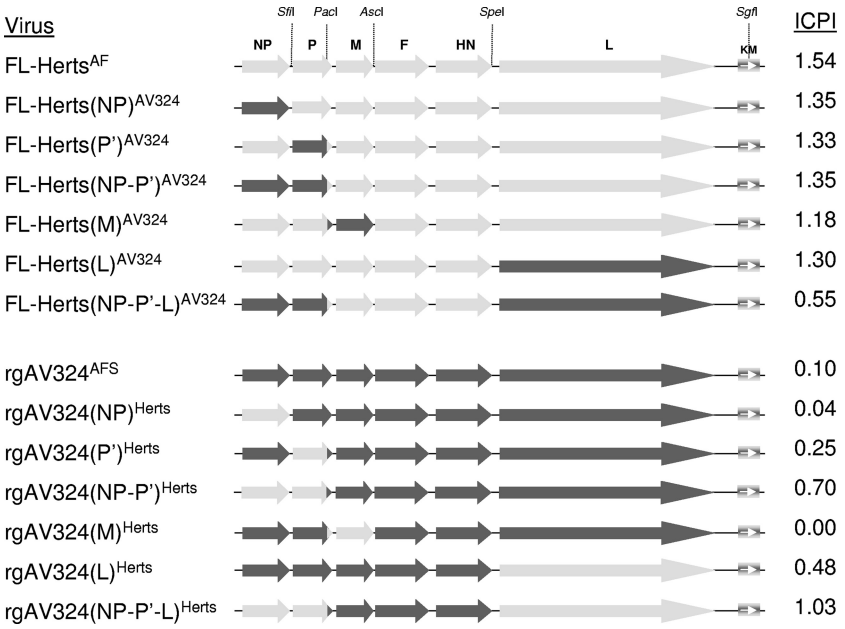

FIG. 1. Schematic illustration of the cloning strategy used to exchange the NP, P, M, and L genes between FL-Herts^{AF} and rgAV324^{AFS}. The virulences of the different recombinant viruses were determined by measuring the intracerebral pathogenicity index (ICPI) in day-old chickens. The maximum ICPI score was 2.0. KM, kanamycin resistance gene positioned in the vector plasmid.

strains of NDV associated with infections of pigeons. Some PPMV-1 strains behave as lentogenic viruses, i.e., they show a low intracerebral pathogenicity index (ICPI) in chickens, despite the presence of an F protein cleavage site motif that is generally associated with virulent viruses (34). In a previous study, we showed that the exchange of the F gene between a low-virulence PPMV-1 virus and a highly virulent virus did not significantly affect the virulences of the chimeric viruses relative to those of their respective parental viruses (16). Thus, the low virulence of some PPMV-1 strains must be determined by other factors. The V, HN, and L proteins of NDV have all been shown to be involved in the virulence of NDV (14, 23, 24, 33, 41, 42, 50, 51). However, little is still known about the mechanisms underlying their function as virulence determinants in NDV strains and especially in PPMV-1 strains.

In this study, we examined the contributions of the NP, P, M, and L proteins to the virulence of NDV. Using reverse genetics, the genes encoding these internal proteins were exchanged between the low-virulence PPMV-1 strain AV324 and the highly virulent NDV strain Herts. The pathogenicities and levels of *in vivo* replication of the chimeric viruses were determined in 1-day-old chickens. Furthermore, we investigated the replication kinetics and plaque sizes of the different chimeric viruses in cell culture monolayers, and we developed an *in vitro* replication assay using cotransfection of plasmids encoding a minigenome that expresses luciferase in the presence of the NP, P, and L proteins. Our results indicate that the virulence of NDV is directly related to the activity of the viral replication complex.

MATERIALS AND METHODS

Cells, viruses, and animals. QM5 cells (4) were grown in QT35 medium (Gibco-BRL/Life Technologies), and DF-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) plus GlutaMAX (Invitrogen). Both media were supplemented with 5% fetal bovine serum and 1% of an antibiotic stock consisting of penicillin (100 units/ml), streptomycin (100 μg/ml), and fungizone (2.5 μg/ml). Both cell lines were grown at 37°C in a 5% CO_2 incubator. The fowlpox recombinant virus fpE-FLT7pol (6) (hereafter called FPV-T7), which expresses the bacteriophage T7 RNA polymerase, was used as recently described (15). The cDNA clone rgAV324 was derived from the low-virulence PPMV-1 strain AV324/96, and the cDNA clone FL-Herts was derived from the virulent NDV strain Herts/33, as previously described (14, 16). In this study, specific-pathogen-free (SPF) chickens were used. Animal experiments were approved by the Ethics Committee for Animal Experiments of the Central Veterinary Institute of Wageningen UR and comply with Dutch law on animal experiments.

Construction of full-length chimeric AV324/Herts antigenomic cDNAs. Chimeric viruses in which either the individual NP, P, M, or L genes, or combinations thereof, were exchanged between the AV324 and Herts strains were generated (Fig. 1). The published nucleotide sequences of the Herts/33 (GenBank accession no. AY741404) and AV324/96 (GenBank accession no. GQ429292) strains were used as guides for the construction of the chimeric viruses.

To swap the replication genes between the plasmids FL-Herts and rgAV324, the unique restriction sites SfiI, PacI, AscI, SpeI, and SgfI were used (Fig. 1). To introduce a unique restriction site for SpeI (position 8101) in the rgAV324^{AF} cDNA (16), site-directed mutagenesis was performed, resulting in rgAV324^{AFS}. The superscript AF represents the introduced restriction sites AscI and FseI, and the superscript AFS represents the introduced restriction sites AscI, FseI, and SpeI. Plasmids FL-Herts^{AF} and rgAV324^{AFS} were digested with SgfI and PacI to simultaneously exchange the NP and P genes, resulting in rgAV324(NP-P′)^{Herts} and FL-Herts(NP-P′)^{AV324}. Because the PacI site is positioned at nucleotide (nt) 2900 in Herts and nt 2906 in AV324, 57 amino acids of the C terminus of the P proteins were not exchanged. Of these 57 amino acids, 7 differ between the two viruses. To exchange the L gene, both full-length cDNA clones were digested with SpeI and SgfI and reciprocally cloned, resulting in rgAV324(L)^{Herts} and FL-Herts(L)^{AV324}. The PacI and SpeI sites were used to simultaneously exchange the NP, P, and L genes, resulting in rgAV324(NP-P′-L)^{Herts} and FL-Herts(NP-P′-L)^{AV324}. To exchange the NP and P genes individually, site-directed mutagenesis was performed to introduce a unique SfiI site in FL-Herts^{AF} (this site is present in AV324 but is lacking in Herts). To exchange the NP gene, the SgfI and SfiI sites were used, resulting in rgAV324(NP)^{Herts} and FL-Herts(NP)^{AV324}, and to exchange the P gene, the SfiI and PacI sites were used, resulting in rgAV324(P′)^{Herts} and FL-Herts(P′)^{AV324}. Finally, the M genes were exchanged, using the PacI and AscI sites, resulting in rgAV324(M)^{Herts} and FL-Herts(M)^{AV324}.

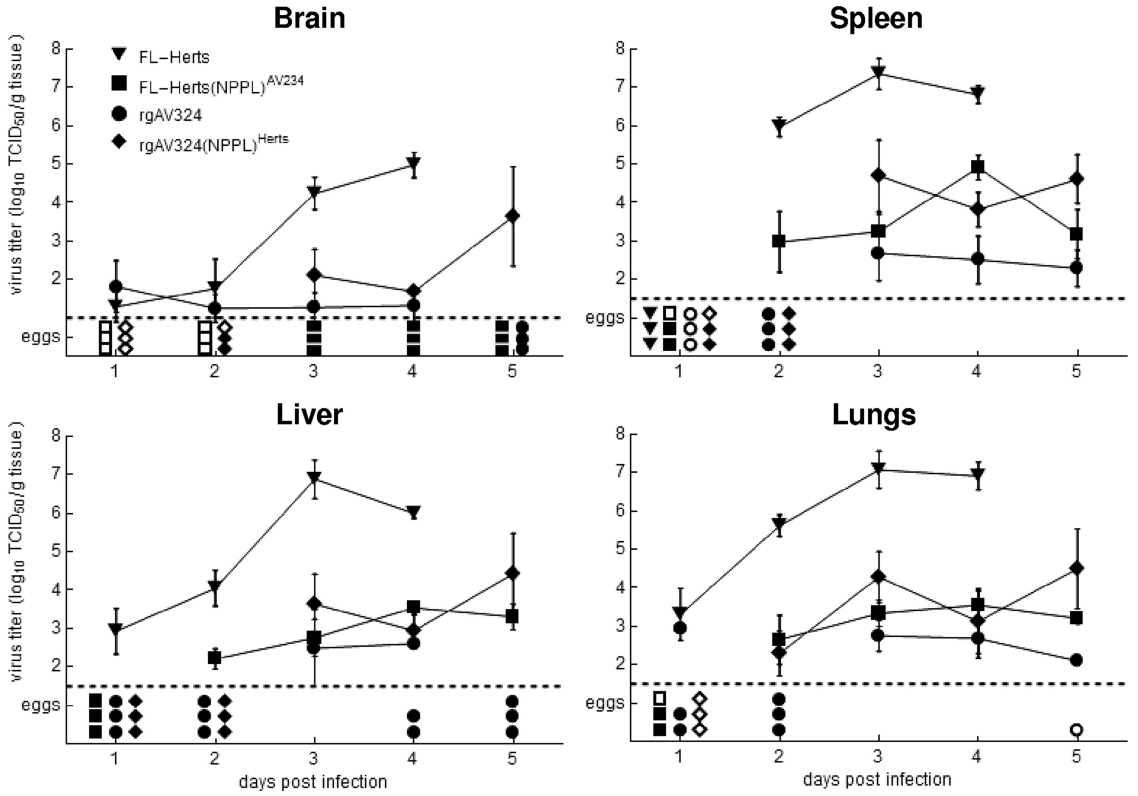

FIG. 2. Viral titers of parental and NP-P′-L chimeric viruses in different organs after intracerebral inoculation of 1-day-old chickens. Each day, three chickens were sacrificed, and brains, spleens, livers, and lungs were collected. Virus titers were determined using QM5 cells and are presented as the average mean virus titers ($\log_{10}$TCID$_{50}$/g tissue). The dotted lines indicate the detection limits of the virus detection assay in QM5 cells. "QM5-negative" samples were additionally tested for the presence of virus by inoculating embryonated eggs. Open symbols indicate that inoculated eggs remained virus negative, and closed symbols indicate that inoculated eggs became virus positive. Because three FL-Herts-infected chickens died 3 days postinfection, there are only four time points for this group. Error bars show standard deviations.

Rescue of virus from cDNA. QM5 cells were infected with FPV-T7 for 1 h and subsequently cotransfected with full-length cDNA constructs and helper plasmids expressing P and L, as previously described (16, 45). The respective helper plasmids of either rgAV324 or FL-Herts were used along with their respective full-length cDNAs to prevent potential heterologous recombination in the transfected cells. After 3 days, the culture supernatant was harvested and inoculated into 9- to 11-day-old embryonated SPF eggs to obtain a virus stock.

Pathogenicity test, HI assay, and reisolation of virus. Determination of the intracerebral pathogenicity index (ICPI) in 1-day-old chickens and the hemagglutination inhibition (HI) assay were performed as described in European Council Directive 92/66/EEC (9). Sera of the chickens that survived the ICPI test were tested in an HI assay to verify that the animals had been infected. In order to check the sequences of the different recombinant viruses after the ICPI tests, brains, livers, and lungs of dead chickens were collected, and virus reisolation in embryonated SPF eggs was performed as previously described (13).

RNA isolation, reverse transcriptase PCR, and sequencing. RNA of the recombinant viruses was isolated, using a High Pure viral RNA kit (Roche Diagnostics). First-strand DNA synthesis was carried out using a SuperScript III reverse transcriptase kit (Invitrogen), and PCR fragments were purified using a High Pure PCR purification kit (Roche Diagnostics). Nucleotide sequencing (primer sequences are available upon request) was carried out using a BigDye Terminator v1.1 cycle sequencing kit and a 3130 genetic analyzer instrument (Applied Biosystems).

Virus quantitation in organs of infected day-old chickens. To compare the replication kinetics of the NP-P′-L chimeric and parental viruses in chickens, 15 1-day-old SPF chicks were intracerebrally inoculated with a 2×10^3 50% tissue culture infective dose (TCID$_{50}$) of virus per chicken. Three birds were sacrificed daily until 5 days postinfection (pi). Brains, spleens, livers, and lungs were collected and homogenized in phosphate-buffered saline (PBS), and the virus titers were determined on QM5 cells, using 10-fold serial dilutions of the cleared homogenates. Average virus titers were calculated, using the Reed and Muench method (48), and are expressed as $\log_{10}$ TCID$_{50}$ per gram of tissue. The dotted lines in Fig. 2 indicate the virus detection limits in QM5 cells. Because undiluted homogenized tissue samples were toxic for the cells, these "QM5-negative" samples were tested for the presence of virus by inoculating undiluted tissue homogenates into embryonated SPF eggs. Open symbols indicate that inoculated eggs remained virus negative, whereas closed symbols indicate that inoculated eggs had become virus positive (Fig. 2).

For statistical analysis, a nonparametric approach based on rank numbers was used, because the numbers of animals were modest and some observations fall below the detection limit. Tests over time were performed, and the group infected with virus A was compared with the group infected with virus B by employing the Wilcoxon rank sum test (the Mann-Whitney test) (8). Per time point, observations were replaced by ranks. The sum of the ranks over time for the group infected with virus A was used as a test statistic, large or small values being critical. The distribution under the null hypothesis of no difference between both infected groups of the test statistic was obtained by simulation, by randomly reshuffling the data over the groups. A 0.05 significance level was used. All calculations were performed with GenStat software (44).

Replication assay. A synthetic DNA containing the T7 RNA polymerase promoter and the Gaussia luciferase (GLuc) gene (in an antisense orientation) flanked at the 5′ side by the trailer region (AV324, nt 14999 to 15192, or Herts, nt 14993 to 15186) and at the 3′ side by the leader region (AV324 or Herts, nt 1 to 121), followed by the hepatitis delta virus ribozyme and the transcription termination signal from bacteriophage T7, was synthesized (GenScript Corporation) and cloned between the LguI and BamHI sites of transcription plasmid pOLTV5 (45), resulting in the minigenome plasmids designated pAV324-GLuc and pHerts-GLuc. The sizes of the minigenomes complied with the rule of six (46). Transcription of plasmid pAV324-GLuc or pHerts-GLuc using T7 RNA polymerase gives rise to genomic (negative-sense) RNA, as has been described previously (46).

QM5 cells were infected with FPV-T7 at a multiplicity of infection (MOI) of

1 for 1 h and subsequently cotransfected with either pAV324-GLuc or pHerts-GLuc and helper plasmids expressing NP, P, and L originating either from the AV324 strain (16) or from the Herts strain (14). The minigenome and its expression plasmids containing NP, P, and L were cotransfected at a ratio of 1.0:1.6:0.8:0.8, respectively, by using FuGENE HD (Roche). For normalization, a plasmid containing the firefly luciferase gene under the control of the human cytomegalovirus (hCMV) immediate-early promoter (a kind gift of Erik de Vries and Xander de Haan, Faculty of Veterinary Medicine, Utrecht, Netherlands) was cotransfected. After 24 h, the expression levels of the secreted (Gaussia) and internal (firefly) luciferase activities were measured, using a luciferase assay kit (Promega) and a GloMax luminometer (Promega). One experiment comprises a triplicate measurement of luciferase expression. A total of six separate experiments were performed. Differences in luciferase expression were statistically analyzed using the Wilcoxon test. Mean differences were considered significant when the P value was less than 0.05.

Analysis of genome replication by quantitation of negative-sense genomic RNA. To investigate the onset and kinetics of virus replication, the relative amounts of negative-sense genomic RNA were determined by quantitative real-time reverse transcriptase PCR (qRRT-PCR) at different time points pi. To set up a qRRT-PCR, the sequences of Herts and AV324 were aligned, using the EMBL-EBI web-based software ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). A PCR primer pair was selected in a homologous region of the L genes such that the primers had no mismatches in either virus. The sequence of the forward primer is 5′-CCCGACCGACTGTGATCTAT-3′, and that of the reverse primer is 5′-GCAGCAAGTTGGATTGCAG-3′. Subsequently, a perfectly matching probe within the same region was chosen and labeled at the 5′ end with 6-carboxyfluorescein (FAM) and at the 3′ end with BHQ1, as follows: 5′-FAM-TGCTAGAGGGGGCATTGAGGGA-BHQ1-3′ (Tib Mobiol).

DF-1 cells were seeded in 24-well plates (Greiner) and infected in duplicate with virus at an MOI of 10. At 2, 4, 6, 8, and 10 h pi (hpi), plates were frozen at −70°C. RNA was extracted from the combined infected cells and the supernatants, using a MagNA Pure LC total nucleic acid isolation kit and a MagNA Pure LC instrument (Roche Applied Science) according to the manufacturer's instructions. Cycling conditions for the qRRT-PCR, using the Mx3005P system (Stratagene), were one cycle at 50°C for 30 min, one cycle at 95°C for 15 min, 45 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. For data analysis, MxPro qPCR software, version 4.10 (Stratagene), was used. The correlation coefficients (R^2) of the standard curves were 0.999 (FL-Herts) and 0.993 (rgAV324). All PCR amplification efficiencies (E) were >0.95. Details of the PCR protocol are available upon request.

Determination of plaque size. Monolayers of QM5 cells and DF-1 cells were infected with the parental and chimeric viruses and incubated for 2 days under an overlay of the Glasgow modification of Eagle medium/Eagle's minimal essential medium (ASG-Lelystad) containing 1% methylcellulose, without the addition of exogenous trypsin. Plaques were visualized by immunological staining, using monoclonal antibody (MAb) Fusie 133 8E12A8C3 (CVI of Wageningen UR) against the NDV F protein and horseradish peroxidase (HRPO)-conjugated polyclonal rabbit anti-mouse Ig (Dako). The average plaque size was determined by measuring the areas of digital images of 12 to 16 discrete plaques per virus (photographed at a magnification of 6.3) using Image-Pro Plus software (MediaCybernetics, Inc.). Differences in plaque size were statistically analyzed using the Wilcoxon test. Mean differences were considered significant when the P value was less than 0.05.

RESULTS

Construction and recovery of recombinant viruses. To study the molecular basis of the low pathogenicities of PPMV-1 strains that contain a fully functional multiple basic amino acid motif in their F protein cleavage sites, we exchanged the genes encoding the internal viral proteins NP, P, M, and L between the nonvirulent PPMV-1 strain AV324 and the highly virulent NDV strain Herts. The amino acid sequence identity of these proteins is 94% for NP, 84% for P, 93% for M, and 95% for L. The cloning strategy that was used to construct the chimeric cDNAs is described in the Materials and Methods section and is illustrated in Fig. 1. Sequence analysis of the chimeric cDNAs confirmed the intended gene exchanges and the absence of any undesired mutations. For virus recovery by means of cotransfection, helper plasmids expressing P and L of either rgAV324 or FL-Herts were used along with their respective full-length cDNAs. All chimeric viruses could be rescued, indicating that the biological functions of these proteins of the Herts and AV324 strains are compatible.

Pathogenicity in day-old chickens. The virulences of the chimeric viruses were assessed by a standard intracerebral pathogenicity test (ICPI) in 1-day-old chickens. Replacement of the internal genes of the Herts strain, either individually or in combination, by those of the AV324 strain resulted in a decrease in virulence. The simultaneous replacement of the genes encoding the complete NP-P′-L replication complex had a significant effect on virulence, as shown by a decrease in the ICPI value from 1.54 for FL-Herts to 0.55 for FL-Herts(NP-P′-L)^{AV324} (Fig. 1). In contrast, although not all replacements of the internal genes of AV324 by those of Herts resulted in an increase in virulence, again the simultaneous replacement of the NP, P, and L genes had a large effect. The exchange resulted in a significant increase in ICPI values from 0.10 for rgAV324 to 1.03 for rgAV324(NP-P′-L)^{Herts} (Fig. 1). These results show that the origin of the complete replication complex from either AV324 or Herts has a major effect on determining the virulence of the chimeric viruses.

***In vivo* replication in day-old chickens.** Because exchanging the complete viral replication complex had the greatest effect on virulence, the levels of *in vivo* replication of these chimeric viruses and their parental viruses were determined in day-old chickens after intracerebral inoculation (Fig. 2). The results showed that the AV324 strain replicated to much lower levels than the Herts strain in all organs examined. In brain tissue, no significant difference in the replication levels of the AV324 recombinant virus that expresses the replication complex of the Herts strain [rgAV324(NP-P′-L)^{Herts}] and that of the parental AV324 strain was observed ($P > 0.05$). However, in liver, lungs, and spleen, replication of rgAV324(NP-P′-L)^{Herts} was significantly enhanced compared to that of rgAV324. Conversely, providing the Herts virus with the AV324 replication complex [FL-Herts(NP-P′-L)^{AV324}] significantly reduced the replicative abilities of the chimeric virus in all tissues examined (Fig. 2). These results strongly suggest that the pathogenicity of avian paramyxoviruses is directly correlated with the level of virus replication in organs of infected animals.

***In vitro* replication assay.** To test whether *in vivo* virus replication correlated with the intrinsic activity of the viral replication complex, we developed an *in vitro* replication assay, using a minigenome that expresses the Gaussia luciferase (GLuc) gene in the presence of the NP, P, and L proteins. Two slightly different minigenomes were used, one based on the leader and trailer sequences of the Herts strain (pHerts-GLuc) and the other on the leader and trailer sequences of the AV324 strain (pAV324-GLuc). Transcription of pAV324-GLuc or pHerts-GLuc using T7 RNA polymerase generates genomic (negative-sense) minigenome RNA. Hence, expression of the GLuc gene is dependent on the conversion of the negative-sense RNA into positive-sense RNA. This process is completely dependent on the viral NP, P, and L proteins, providing a suitable assay to test the roles of the individual proteins in minigenome replication activity.

The *in vitro* replication assay revealed that the two minigenomes behaved similarly, exhibiting comparable levels of re-

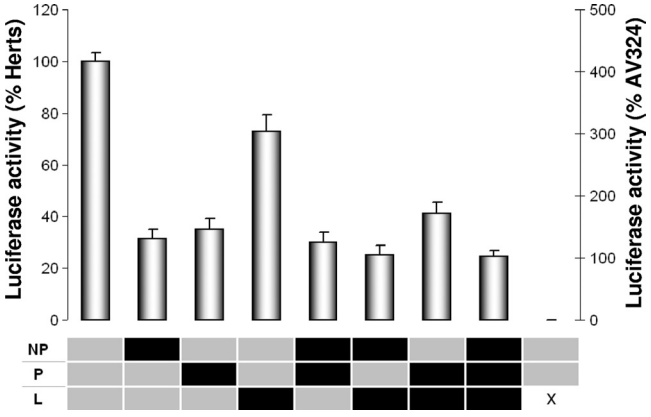

FIG. 3. Relative luciferase expression levels after cotransfection of FPV-T7-infected QM5 cells with the viral minigenome plasmid (containing the leader and trailer sequences of Herts that flank the Gluc reporter gene) and plasmids expressing NP, P, and L of Herts (gray) or AV324 (black). The background level of luciferase activity was determined by omitting the L plasmid (X). The graph shows the average values obtained in six separate experiments. On the left *y* axis, values for luciferase activities relative to Herts are given, and on the right *y* axis, values for luciferase activities relative to AV324 are shown. Error bars represents standard errors of the means (SEMs).

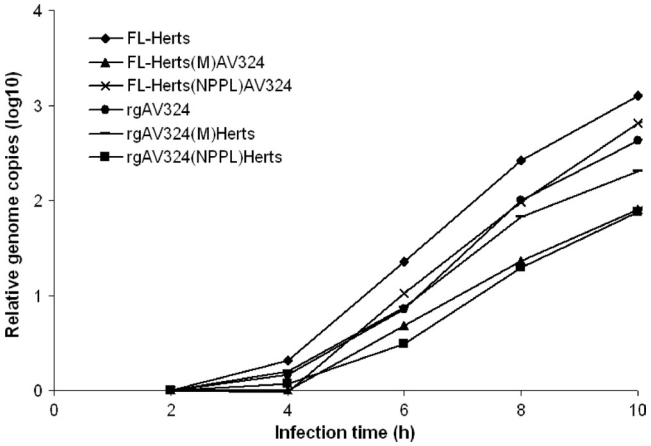

FIG. 4. Onset and kinetics of negative-sense genomic RNA replication in the first 10 h of infection. DF-1 cells were infected at an MOI of 10, and replication was determined by qRRT-PCR. Results are averages for duplicate infections.

porter gene expression when driven by both the AV324 replication proteins and those of the Herts virus (data not shown). This indicates that the nucleotide differences in the leader and trailer sequences that code for the genomic (3-nt difference) and antigenomic (4-nt difference) promoter (31) of AV324 and Herts did not affect the efficiency of viral replication. Therefore, only the results obtained with the pHerts-GLuc minigenome are shown. The results revealed that the activity of the AV324 replication complex is significantly lower than that of the Herts replication complex (Fig. 3). Furthermore, none of the possible combinations of the NP, P, and L proteins of AV324 and Herts reached the 100% level obtained with the Herts replication proteins only. These observations suggest that optimal replication is not determined by one or two proteins but by the combined action of all three replication proteins.

Viral genome replication. To investigate the onset and kinetics of viral genome replication of the recombinant viruses in cell culture monolayers, a qRRT-PCR that specifically detects negative-strand viral RNA was used. To this end, DF-1 cells were infected at an MOI of 10, and at different time points up to 10 hpi, the amount of genomic RNA was determined. The results showed that the genome of FL-Herts replicated at a higher rate than that of rgAV324 (Fig. 4). When the Herts virus was provided with the AV324 replication complex, the replication kinetics were similar to those of rgAV324. Unexpectedly, however, the recombinant virus in which the replication genes of AV324 were replaced by those of Herts [rgAV324(NP-P′-L)^{Herts}] showed the lowest replication efficiency of all viruses tested. Because several studies have shown that the M protein may affect transcription and/or replication, we also examined the recombinant viruses in which only the M gene was exchanged. The results showed that the kinetics of FL-Herts(M)^{AV324} were very similar to those of rgAV324(NP-P′-L)^{Herts} (Fig. 4), suggesting that the specific combination of the M protein of AV324 and the NP-P′-L complex of Herts results in a decreased replication rate. This effect also seems to affect virulence, since the introduction of the AV324 M protein in Herts resulted in a reduction of the ICPI value from 1.54 to 1.18 (Fig. 1). The kinetics of genome replication of rgAV324 was not increased by the M protein of Herts, and no significant effect on virulence was noted [cf. rgAV324 and rgAV324(M)^{Herts}], indicating that the effect is nonreciprocal.

Plaque size. In order to examine the replication of the different viruses in tissue culture cells in another way, we determined their plaque sizes in monolayers of quail-derived QM5 cells and chicken-derived DF-1 cells at 48 hpi (Fig. 5). Relative to FL-Herts, rgAV324 showed a significantly smaller plaque size in QM5 and in DF-1 cells. Furthermore, smaller plaque sizes in both cell types were also observed when the Herts virus was provided with the M protein of AV324 or the replication complex of AV324. When the AV324 virus expressed the M protein or the replication complex of Herts, QM5 cells and DF-1 cells showed contrasting results with regard to plaque size. While the plaque sizes were smaller in QM5 cells, larger plaques were observed in DF-1 cells (Fig. 5).

DISCUSSION

The results of this study show that all three proteins that make up the viral replication complex (NP, P, and L) play a significant role in determining the virulence of NDV. By exchanging the replication genes simultaneously, the virulent Herts virus was significantly attenuated, whereas the low-virulence AV324 strain became much more virulent. All individual replication proteins have their own contribution but act synergistically when all three together are exchanged. The matrix (M) protein of AV324 showed a distinct effect on virulence in the Herts background, probably by interacting with the viral replication complex. However, this effect was not reciprocal, since the M protein of Herts lacked the ability to increase the virulence of AV324.

The difference in virulence between AV324 and Herts seems to be directly related to the efficiency of *in vivo* viral replica-

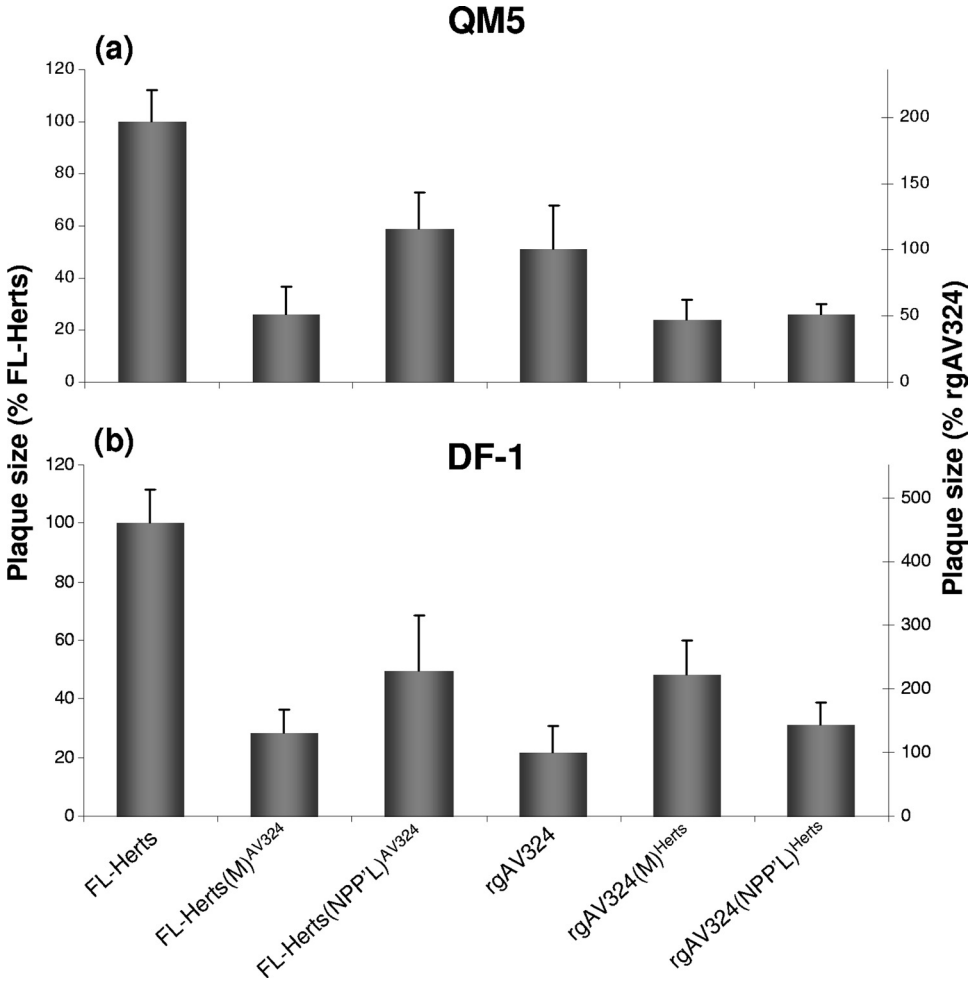

FIG. 5. Relative plaque sizes of the recombinant viruses in QM5 (a) and DF-1 (b) cells 48 h postinfection. Plaques were visualized by immunological staining using a MAb against the NDV F protein. The average plaque size was determined by measuring the areas of at least 12 plaques per virus with Image-Pro Plus software (Media Cybernetics, Inc.). On the left *y* axis, plaque sizes relative to those of FL-Herts are given, and on the right *y* axis, plaque sizes relative to those of rgAV324 are shown. Error bars represent standard deviations.

tion. After the genes encoding the entire NP-P-L replication complex were exchanged, the virus titers of the virulent Herts strain in day-old chickens were decreased in all organs examined, whereas those of the avirulent AV324 strain were increased in three of the four organs tested (Fig. 2). Lentogenic NDV strains are generally unable to spread systemically when inoculated intracerebrally. Because these viruses lack the multibasic cleavage motif in their F proteins, activation requires trypsin-like proteases that are apparently not present in neuronal tissue. Furthermore, replication of these viruses is often limited to the inoculation site (39, 51, 60). The titers of the AV324 strain in brain tissue gradually decreased over time, but the virus was still able to spread to secondary organs (Fig. 2). However, this systemic infection did not sicken its host drastically, as evidenced by the virus's ICPI of 0.10 (Fig. 1) and the absence of clinical signs (data not shown). Thus, low-virulence PPMV-1 isolates may be perfectly adapted to their hosts by being able to spread systemically due to their typical velogenic F cleavage site motif while replicating at a relatively low level.

The differences in efficiencies of the viral replication complexes were confirmed in an *in vitro* replication assay (Fig. 3). Optimal replication was observed when all three replication proteins originated from the Herts strain. This finding matches the results of the ICPI tests (Fig. 1). However, none of the possible combinations of the NP, P, and L proteins of AV324 and Herts reached the level of replication obtained with only Herts replication proteins. One of the possible explanations may be that the individual Herts replication proteins are inherently more active but that optimal activity is dependent on the presence of the cognate interaction partners. The amino acid sequences of the protein domains responsible for the interaction of the NP and P proteins (26) differ between Herts and AV324. Furthermore, although not well identified for NDV, the interaction domains responsible for the P-L interaction presumably also differ between the two viruses, as they have been shown to differ among several other paramyxoviruses (20, 22, 27, 43, 58). Since these complexes are essential for transcription and replication of the viral genome (28), they probably do not function optimally in a heterologous constitution.

The discrepancy between the *in vivo* results (Fig. 1 and 2)

and the *in vitro* results (Fig. 4 and 5) might be explained by the role of the M protein. While the M protein is considered to be the central organizer of viral morphogenesis (28), several studies have shown that in addition, it is involved in regulating viral RNA synthesis (19, 25, 49). With NP as its most likely binding partner (25, 40), the M protein associates with the nucleocapsid (19, 29, 56). Upon viral entry into the target cell, the nucleocapsid dissociates from the M protein and is released into the cytoplasm, where transcription can occur. The interaction of the M protein with the nucleocapsid might affect transcription and have a subsequent effect on replication. Furthermore, this interaction may be strain specific and nonreciprocal and might explain the relatively low replication rate (Fig. 4) and small plaque size (Fig. 5) of rgAV324(NP-P′-L)$^{\text{Herts}}$, since similar results were found for FL-Herts(M)$^{\text{AV324}}$. Another possible explanation for our results is that the association of the M protein with host cell factors might differ between the two strains. During the infection cycle, the M protein of NDV and other paramyxoviruses traffics between the cytoplasm and the nucleus (21). Early during infection, the M protein resides primarily in the nucleus, while later during infection M is localized mainly in the cytoplasm (7, 21). It has been suggested that the function of M in the nucleus relates to inhibition of host cell functions (1, 18), although this has not yet been confirmed for NDV. The nuclear localization signals (7) and the proposed viral late-domain core sequence necessary for budding, ^{24}FPIV^{27} (52), do not differ between Herts and AV324 M proteins.

The involvement of the NP, P, and L proteins in NDV virulence has been examined before (51). In that study, chimeric viruses were generated by exchanging genes between the lentogenic strain LaSota and the mesogenic strain Beaudette C, both classified as members of lineage 2 or genotype II in the avian paramyxovirus type 1 group (2). Surprisingly, a recombinant Beaudette C virus that contained the L gene of LaSota replicated at a higher level and was more virulent than its parental virus. However, no effect was found for the NP and P proteins. These results differ from our findings, which show that all three replication proteins are associated with virulence. An explanation for this difference might be that LaSota and Beaudette C belong to the same phylogenetic lineage and genotype, whereas the strains used in this study belong to different lineages and genotypes. Herts is classified as a member of lineage 3b (or genotype IV), and AV324 belongs to lineage 4b (or genotype VI) (2). Furthermore, Herts is a chicken-derived strain, and AV324 is of pigeon origin.

The molecular mechanism for the relationship between the level of replication of a virus and its pathogenesis is not fully understood. It is conceivable that higher levels of RNA synthesis lead to higher levels of viral replication and thus to more virus production. This may overwhelm the host immune response, causing enhanced pathogenesis. A correlation between virulence and the efficiency of viral replication has been observed before. It has, for instance, been reported that reduced levels of RNA synthesis are associated with reduced virulence of NDV (30). For several other paramyxoviruses, such as measles virus (5, 57), respiratory syncytial virus, and parainfluenza virus (35, 54), it has been described that determinants of virus attenuation are associated with mutations in the P and L genes.

Due to the use of the PacI cloning site, the exchange of the P genes actually resulted in the exchange of chimeric P proteins in which 7 of the C-terminal 57 amino acids that differ between the P proteins of Herts and AV324 are still similar to those of the backbone strain. Exchanging the chimeric P genes had only a limited effect on virulence [ICPI = 1.33 for FL-Herts(P)′$^{\text{AV324}}$ and ICPI = 0.25 for rgAV324(P′)$^{\text{Herts}}$]. However, we cannot completely exclude the possibility that exchanging the complete P protein may have had a different effect on its function and virulence.

In conclusion, this study shows that in addition to the F, V, HN, and L proteins, the NP, P, and L protein complex contributes to the virulence of NDV. Additional studies will be required to elucidate whether the proteins have an effect on viral transcription, replication, or both. Altogether, these observations illustrate that the virulence of NDV is a complex trait determined by multiple genetic factors. Furthermore, the degree to which these factors are involved in NDV virulence seems to be strain and cell type dependent.

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REFERENCES

1. **Ahmed, M., M. O. McKenzie, S. Puckett, M. Hojnacki, L. Poliquin, and D. S. Lyles.** 2003. Ability of the matrix protein of vesicular stomatitis virus to suppress beta interferon gene expression is genetically correlated with the inhibition of host RNA and protein synthesis. J. Virol. **77:**4646–4657.
2. **Aldous, E. W., J. K. Mynn, J. Banks, and D. J. Alexander.** 2003. A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. Avian Pathol. **32:**239–256.
3. **Alexander, D. J., and R. E. Gough.** 2003. Newcastle disease, other avian paramyxoviruses and pneumovirus infections, p. 63–92. *In* Y. M. Saif, H. J. Barnes, A. M. Fadly, J. R. Glisson, L. R. McDougald, and D. E. Swayne (ed.), Diseases of poultry, 11 ed. Iowa State University Press, Ames, IA.
4. **Antin, P. B., and C. P. Ordahl.** 1991. Isolation and characterization of an avian myogenic cell line. Dev. Biol. **143:**111–121.
5. **Bankamp, B., S. P. Kearney, X. Liu, W. J. Bellini, and P. A. Rota.** 2002. Activity of polymerase proteins of vaccine and wild-type measles virus strains in a minigenome replication assay. J. Virol. **76:**7073–7081.
6. **Britton, P., P. Green, S. Kottier, K. L. Mawditt, Z. Penzes, D. Cavanagh, and M. A. Skinner.** 1996. Expression of bacteriophage T7 RNA polymerase in avian and mammalian cells by a recombinant fowlpox virus. J. Gen. Virol. **77:**963–967.
7. **Coleman, N. A., and M. E. Peeples.** 1993. The matrix protein of Newcastle disease virus localizes to the nucleus via a bipartite nuclear localization signal. Virology **195:**596–607.
8. **Conover, W. J.** 1980. Practical nonparametric statistics, 2 ed. John Wiley and Sons, New York, NY.
9. **Council of the European Communities.** 1992. Council directive 92/66/EEC of 14 July 1992 introducing community measures for the control of Newcastle disease. Off. J. Eur. Union **L260:**1–20.
10. **Curran, J.** 1996. Reexamination of the Sendai virus P protein domains required for RNA synthesis: a possible supplemental role for the P protein. Virology **221:**130–140.
11. **Curran, J., J. B. Marq, and D. Kolakofsky.** 1995. An N-terminal domain of the Sendai paramyxovirus P protein acts as a chaperone for the NP protein during the nascent chain assembly step of genome replication. J. Virol. **69:**849–855.
12. **Curran, J., J. B. Marq, and D. Kolakofsky.** 1992. The Sendai virus nonstructural C proteins specifically inhibit viral mRNA synthesis. Virology **189:**647–656.
13. **de Leeuw, O. S., L. Hartog, G. Koch, and B. P. Peeters.** 2003. Effect of fusion protein cleavage site mutations on virulence of Newcastle disease virus:

non-virulent cleavage site mutants revert to virulence after one passage in chicken brain. J. Gen. Virol. **84:**475–484.
14. **de Leeuw, O. S., G. Koch, L. Hartog, N. Ravenshorst, and B. P. Peeters.** 2005. Virulence of Newcastle disease virus is determined by the cleavage site of the fusion protein and by both the stem region and globular head of the haemagglutinin-neuraminidase protein. J. Gen. Virol. **86:**1759–1769.
15. **Dortmans, J. C., C. M. Fuller, E. W. Aldous, P. J. Rottier, and B. P. Peeters.** 2010. Two genetically closely related pigeon paramyxovirus type 1 (PPMV-1) variants with identical velogenic fusion protein cleavage sites but with strongly contrasting virulence. Vet. Microbiol. **143:**139–144.
16. **Dortmans, J. C., G. Koch, P. J. M. Rottier, and B. P. H. Peeters.** 2009. Virulence of pigeon paramyxovirus type 1 does not always correlate with the cleavability of its fusion protein. J. Gen. Virol. **90:**2746–2750.
17. **Finke, S., R. Mueller-Waldeck, and K. K. Conzelmann.** 2003. Rabies virus matrix protein regulates the balance of virus transcription and replication. J. Gen. Virol. **84:**1613–1621.
18. **Ghildyal, R., A. Ho, and D. A. Jans.** 2006. Central role of the respiratory syncytial virus matrix protein in infection. FEMS Microbiol. Rev. **30:**692–705.
19. **Ghildyal, R., J. Mills, M. Murray, N. Vardaxis, and J. Meanger.** 2002. Respiratory syncytial virus matrix protein associates with nucleocapsids in infected cells. J. Gen. Virol. **83:**753–757.
20. **Hamaguchi, M., T. Yoshida, K. Nishikawa, H. Naruse, and Y. Nagai.** 1983. Transcriptive complex of Newcastle disease virus. I. Both L and P proteins are required to constitute an active complex. Virology **128:**105–117.
21. **Harrison, M. S., T. Sakaguchi, and A. P. Schmitt.**14 April 2010, posting date. Paramyxovirus assembly and budding: building particles that transmit infections. Int. J. Biochem. Cell Biol.[Epub ahead of print.] doi:10.1016/j.biocel.2010.04.005.
22. **Horikami, S. M., J. Curran, D. Kolakofsky, and S. A. Moyer.** 1992. Complexes of Sendai virus NP-P and P-L proteins are required for defective interfering particle genome replication in vitro. J. Virol. **66:**4901–4908.
23. **Huang, Z., S. Krishnamurthy, A. Panda, and S. K. Samal.** 2003. Newcastle disease virus V protein is associated with viral pathogenesis and functions as an alpha interferon antagonist. J. Virol. **77:**8676–8685.
24. **Huang, Z., A. Panda, S. Elankumaran, D. Govindarajan, D. D. Rockemann, and S. K. Samal.** 2004. The hemagglutinin-neuraminidase protein of Newcastle disease virus determines tropism and virulence. J. Virol. **78:**4176–4184.
25. **Iwasaki, M., M. Takeda, Y. Shirogane, Y. Nakatsu, T. Nakamura, and Y. Yanagi.** 2009. The matrix protein of measles virus regulates viral RNA synthesis and assembly by interacting with the nucleocapsid protein. J. Virol. **83:**10374–10383.
26. **Jahanshiri, F., M. Eshaghi, and K. Yusoff.** 2005. Identification of phosphoprotein:phosphoprotein and phosphoprotein:nucleocapsid protein interaction domains of the Newcastle disease virus. Arch. Virol. **150:**611–618.
27. **Kingston, R. L., D. J. Hamel, L. S. Gay, F. W. Dahlquist, and B. W. Matthews.** 2004. Structural basis for the attachment of a paramyxoviral polymerase to its template. Proc. Natl. Acad. Sci. U. S. A. **101:**8301–8306.
28. **Lamb, R. A., and G. D. Parks.** 2007. Paramyxoviridae: the viruses and their replication, p. 1449–1496. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
29. **Li, D., D. A. Jans, P. G. Bardin, J. Meanger, J. Mills, and R. Ghildyal.** 2008. Association of respiratory syncytial virus M protein with viral nucleocapsids is mediated by the M2-1 protein. J. Virol. **82:**8863–8870.
30. **Madansky, C. H., and M. A. Bratt.** 1981. Noncytopathic mutants of Newcastle disease virus are defective in virus-specific RNA synthesis. J. Virol. **37:**317–327.
31. **Marcos, F., L. Ferreira, J. Cros, M. S. Park, T. Nakaya, A. Garcia-Sastre, and E. Villar.** 2005. Mapping of the RNA promoter of Newcastle disease virus. Virology **331:**396–406.
32. **Mayo, M. A.** 2002. A summary of taxonomic changes recently approved by ICTV. Arch. Virol. **147:**1655–1663.
33. **Mebatsion, T., S. Verstegen, L. T. De Vaan, A. Romer-Oberdorfer, and C. C. Schrier.** 2001. A recombinant Newcastle disease virus with low-level V protein expression is immunogenic and lacks pathogenicity for chicken embryos. J. Virol. **75:**420–428.
34. **Meulemans, G., T. P. van den Berg, M. Decaesstecker, and M. Boschmans.** 2002. Evolution of pigeon Newcastle disease virus strains. Avian Pathol. **31:**515–519.
35. **Murphy, B. R., and P. L. Collins.** 2002. Live-attenuated virus vaccines for respiratory syncytial and parainfluenza viruses: applications of reverse genetics. J. Clin. Invest. **110:**21–27.
36. **Nagai, Y., H. D. Klenk, and R. Rott.** 1976. Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. Virology **72:**494–508.
37. **Ogasawara, T., B. Gotoh, H. Suzuki, J. Asaka, K. Shimokata, R. Rott, and Y. Nagai.** 1992. Expression of factor X and its significance for the determination of paramyxovirus tropism in the chick embryo. EMBO J. **11:**467–472.
38. **Ogino, T., M. Iwama, Y. Ohsawa, and K. Mizumoto.** 2003. Interaction of cellular tubulin with Sendai virus M protein regulates transcription of viral genome. Biochem. Biophys. Res. Commun. **311:**283–293.
39. **Panda, A., Z. Huang, S. Elankumaran, D. D. Rockemann, and S. K. Samal.** 2004. Role of fusion protein cleavage site in the virulence of Newcastle disease virus. Microb. Pathog. **36:**1–10.
40. **Pantua, H. D., L. W. McGinnes, M. E. Peeples, and T. G. Morrison.** 2006. Requirements for the assembly and release of Newcastle disease virus-like particles. J. Virol. **80:**11062–11073.
41. **Park, M. S., A. Garcia-Sastre, J. F. Cros, C. F. Basler, and P. Palese.** 2003. Newcastle disease virus V protein is a determinant of host range restriction. J. Virol. **77:**9522–9532.
42. **Park, M. S., M. L. Shaw, J. Munoz-Jordan, J. F. Cros, T. Nakaya, N. Bouvier, P. Palese, A. Garcia-Sastre, and C. F. Basler.** 2003. Newcastle disease virus (NDV)-based assay demonstrates interferon-antagonist activity for the NDV V protein and the Nipah virus V, W, and C proteins. J. Virol. **77:**1501–1511.
43. **Parks, G. D.** 1994. Mapping of a region of the paramyxovirus L protein required for the formation of a stable complex with the viral phosphoprotein P. J. Virol. **68:**4862–4872.
44. **Payne, R. W., S. A. Harding, D. A. Murray, D. M. Soutar, D. B. Baird, A. I. Glaser, I. C. Channing, S. J. Welham, A. R. Gilmour, R. Thompson, and R. Webster.** 2009. GenStat release 12 reference manual. VSN International, Hemel Hempstead, United Kingdom.
45. **Peeters, B. P., O. S. de Leeuw, G. Koch, and A. L. Gielkens.** 1999. Rescue of Newcastle disease virus from cloned cDNA: evidence that cleavability of the fusion protein is a major determinant for virulence. J. Virol. **73:**5001–5009.
46. **Peeters, B. P., Y. K. Gruijthuijsen, O. S. de Leeuw, and A. L. Gielkens.** 2000. Genome replication of Newcastle disease virus: involvement of the rule-of-six. Arch. Virol. **145:**1829–1845.
47. **Poch, O., B. M. Blumberg, L. Bougueleret, and N. Tordo.** 1990. Sequence comparison of five polymerases (L proteins) of unsegmented negative-strand RNA viruses: theoretical assignment of functional domains. J. Gen. Virol. **71:**1153–1162.
48. **Reed, L. J., and H. A. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27:**493–497.
49. **Reuter, T., B. Weissbrich, S. Schneider-Schaulies, and J. Schneider-Schaulies.** 2006. RNA interference with measles virus N, P, and L mRNAs efficiently prevents and with matrix protein mRNA enhances viral transcription. J. Virol. **80:**5951–5957.
50. **Römer-Oberdörfer, A., J. Veits, O. Werner, and T. C. Mettenleiter.** 2006. Enhancement of pathogenicity of Newcastle disease virus by alteration of specific amino acid residues in the surface glycoproteins F and HN. Avian Dis. **50:**259–263.
51. **Rout, S. N., and S. K. Samal.** 2008. The large polymerase protein is associated with the virulence of Newcastle disease virus. J. Virol. **82:**7828–7836.
52. **Schmitt, A. P., G. P. Leser, E. Morita, W. I. Sundquist, and R. A. Lamb.** 2005. Evidence for a new viral late-domain core sequence, FPIV, necessary for budding of a paramyxovirus. J. Virol. **79:**2988–2997.
53. **Sidhu, M. S., J. P. Menonna, S. D. Cook, P. C. Dowling, and S. A. Udem.** 1993. Canine distemper virus L gene: sequence and comparison with related viruses. Virology **193:**50–65.
54. **Skiadopoulos, M. H., A. P. Durbin, J. M. Tatem, S. L. Wu, M. Paschalis, T. Tao, P. L. Collins, and B. R. Murphy.** 1998. Three amino acid substitutions in the L protein of the human parainfluenza virus type 3 cp45 live attenuated vaccine candidate contribute to its temperature-sensitive and attenuation phenotypes. J. Virol. **72:**1762–1768.
55. **Steward, M., I. B. Vipond, N. S. Millar, and P. T. Emmerson.** 1993. RNA editing in Newcastle disease virus. J. Gen. Virol. **74:**2539–2547.
56. **Suryanarayana, K., K. Baczko, V. ter Meulen, and R. R. Wagner.** 1994. Transcription inhibition and other properties of matrix proteins expressed by M genes cloned from measles viruses and diseased human brain tissue. J. Virol. **68:**1532–1543.
57. **Takeda, M., A. Kato, F. Kobune, H. Sakata, Y. Li, T. Shioda, Y. Sakai, M. Asakawa, and Y. Nagai.** 1998. Measles virus attenuation associated with transcriptional impediment and a few amino acid changes in the polymerase and accessory proteins. J. Virol. **72:**8690–8696.
58. **Tarbouriech, N., J. Curran, C. Ebel, R. W. Ruigrok, and W. P. Burmeister.** 2000. On the domain structure and the polymerization state of the Sendai virus P protein. Virology **266:**99–109.
59. **Vidal, S., and D. Kolakofsky.** 1989. Modified model for the switch from Sendai virus transcription to replication. J. Virol. **63:**1951–1958.
60. **Wakamatsu, N., D. J. King, B. S. Seal, B. P. Peeters, and C. C. Brown.** 2006. The effect on pathogenesis of Newcastle disease virus LaSota strain from a mutation of the fusion cleavage site to a virulent sequence. Avian Dis. **50:**483–488.